

A novel cytosolic class I antigen-processing pathway for endoplasmic-reticulum-targeted proteins

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Proteins bearing an endoplasmic reticulum (ER) leader are inserted into the ER followed by cleavage of the signal peptide. Major histocompatibility complex class I-restricted T-cell epitopes can be generated from these proteins by the proteasome after retrotranslocation into the cytosol. Here, we show that an HLA-A*0201-restricted epitope from prostate stem cell antigen contains the cleavage site of the ER signal peptidase. The resulting cleavage products fail to bind to HLA-A*0201 and are not recognized by T lymphocytes. As processing of prostate stem cell antigen by signal peptidase occurs immediately after co-translational insertion, the epitope must be processed from polypeptides that have never reached the ER. The processing of this epitope depends on the proteasome and the transporter associated with antigen processing and shows a novel pathway of class I processing that relies on the failure of ER-targeted proteins to reach their target compartment.

Keywords: endoplasmic reticulum; antigen processing; major histocompatibility complex class I; signal peptidase; cytotoxic T lymphocytes

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INTRODUCTION

Tumour cells and viruses with envelopes express glycoproteins that are co-translationally inserted into the endoplasmic reticulum (ER). Numerous major histocompatibility complex (MHC) class I-restricted epitopes are derived from glycoproteins that are glycosylated in the ER lumen. Initially, it was unknown why the class I-restricted presentation of epitopes from ER-targeted glycoproteins was dependent on the transporter associated with antigen processing (TAP), which transports peptides from the cytosol into the lumen of the ER (Hammond *et al*, 1995; Skipper *et al*, 1996). Subsequently, it was discovered that ER-resident proteins are retrotranslocated through the translocon into the cytoplasm and degraded by the proteasome in a process called ER-associated protein degradation (ERAD). In elegant studies on epitopes from tyrosinase (Mosse *et al*, 1998), hepatitis virus envelope glycoprotein E1 (Selby *et al*, 1999), HIV-1 envelope glycoprotein (Ferris *et al*, 1999) and influenza virus nucleoprotein (Bacik *et al*, 1997), it was shown that an asparagine residue in the epitopes was first glycosylated in the ER and then, after retrotranslocation, deglycosylated in the cytosol. Deglycosylation by peptidyl-N-glycanase leads to deamidation of asparagine to aspartate within the epitopes, which is then translocated back into the ER by TAP and loaded onto MHC class I.

Recently, we have identified the human leukocyte antigen (HLA)-A*0201-restricted cytotoxic T lymphocyte (CTL) epitope PSCA_{14–22} within the prostate-specific protein prostate stem cell antigen (PSCA; Dannull *et al*, 2000). PSCA is expressed in the basal cell epithelium of the prostate, and in cancer cells derived from there, which qualifies PSCA as a target of immunotherapy against prostate carcinoma. PSCA bears an ER signal sequence at its amino terminus and a consensus site for attachment of a glycosyl-phosphatidyl-inositol anchor at its carboxyl terminus (Reiter *et al*, 1998). The predicted molecular mass of PSCA is 12.9 kDa, but the apparent molecular mass is about 24 kDa due to N-glycosylation within the ER. Curiously, the nonameric epitope PSCA_{14–22} encompassing the sequence ALQPGTALL is predicted to be cleaved by the ER signal peptidase before the two C-terminal

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leucine residues that harbour the hydrophobic anchor residue for binding to HLA-A*0201. Here, we show experimentally that these two leucine residues are indeed located at the N terminus of a secreted PSCA variant, thus confirming the predicted signal peptide cleavage site. This cleavage destroys the epitope, implying that the PSCA polypeptides from which PSCA_{14–22} is processed have never entered the ER. Therefore, PSCA_{14–22} is processed in a new pathway that generates epitopes from polypeptides that are meant to be inserted into the ER but that are degraded by the proteasome in the cytoplasm before they reach the lumen of the ER.

RESULTS AND DISCUSSION

Identification of the mature amino terminus of PSCA

To predict the location of the ER signal peptide cleavage site in the amino-acid sequence, we analysed the human PSCA sequence using the Signal P-NN program (www.cbs.dtu.dk). For PSCA, a cleavage site was predicted between A₂₁ and L₂₂, which would be within the nonameric epitope PSCA_{14–22} (ALQPGTA'LL) between the seventh and eighth residues (Fig 1A). As ligands of HLA-A*0201 are normally 9 amino-acid residues long and rely on a hydrophobic residue as a C-terminal anchor, the removal of the two leucine residues from the C terminus was likely to destroy the epitope.

To determine experimentally the processed N terminus of the leader-less PSCA, we generated an expression construct for PSCA, in which the C-terminal consensus site for attachment of a glycosyl-phosphatidyl-inositol anchor was removed and replaced by a haemagglutinin (HA) tag with the consequence that the resulting PSCA-HA protein is secreted. This construct was used to generate stable transfectants of the human embryonic kidney cell line HEK293, and a clone named HEK293-PSCA-HA was selected for high PSCA-HA expression. Western blot analysis showed that these cells expressed the highly glycosylated PSCA-HA protein with the apparent molecular weight of 20 kDa that was reduced by deglycosylation to about 10 kDa (data not shown). This protein was readily secreted into the supernatant of HEK293-PSCA-HA cells and could be immunoprecipitated in large amounts. The purified PSCA-HA was in-gel digested with trypsin, and tryptic peptides were eluted for analysis by mass spectrometry. Electrospray ionization mass spectrometry on two independent preparations showed the peptide LLCYSCK as the most N-terminal peptide with high abundance (ion score of 31; Fig 1B). The N-terminal amino acid of this peptide is an alanine, which is a residue that does not represent a trypsin cleavage site. Hence, it is likely that the N terminus of this peptide is defined by cleavage of the ER signal peptidase. In one of the two experiments, we also detected a longer peptide with about 100-fold lower abundance that started at position 15 of PSCA, that is, at position 2 of the PSCA_{14–22} epitope (Fig 1C). This cleavage also bears an alanine

residue in the P1 position and hence is not likely to be generated by a trypsin residue. For position 15, a minor ER signal peptidase cleavage site is predicted, but the respective fragment was detected with a low ion score and only in one of the two experiments.

To identify the N terminus of PSCA-HA by using an independent method, automated N-terminal Edman degradation was carried out. Edman sequencing showed that PSCA-HA started at residue 21 with the N-terminal amino acids LLCYS (Fig 1D). As no evidence for an N terminus starting from residue 15 was provided by Edman degradation, this cleavage seems to be minor and of questionable biological importance.

Consequences of signal peptidase cleavage

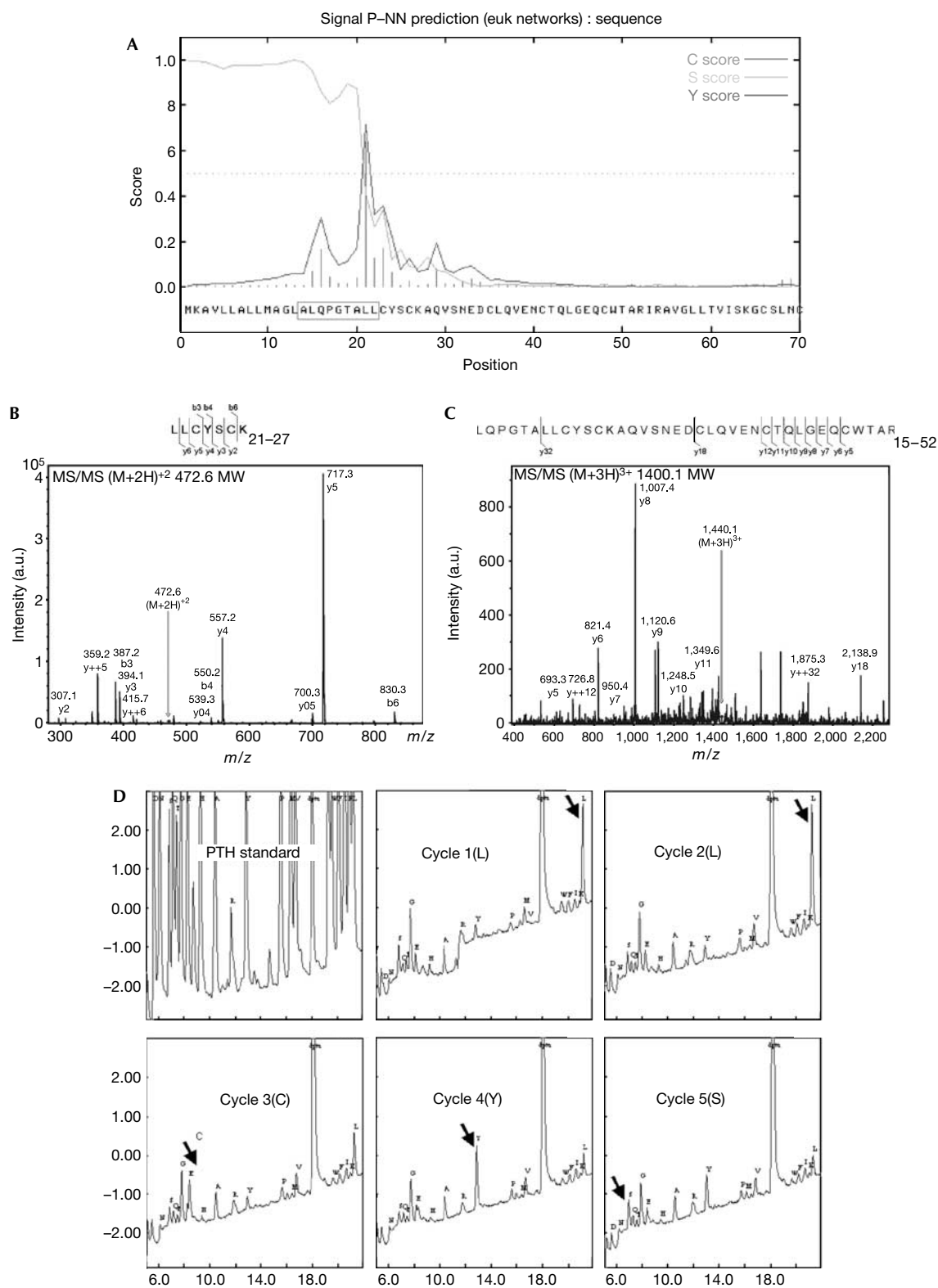
Cleavage of PSCA by signal peptidase at positions 15 and 20 would result in the octamer LQPGTALL and the heptamer ALQPGTA, respectively. For these two peptides, we carried out HLA-A*0201 stabilization assays with TAP-deficient T2 cells. As shown in Fig 2A, the PSCA heptamer was unable to stabilize HLA-A*0201, whereas the PSCA octamer stabilized HLA-A*0201 at 25 °C to a low degree. By contrast, the HLA-A*0201 cell-surface expression achieved by incubation with the PSCA nonamer, and as a positive control the influenza virus matrix epitope M1, was approximately sixfold higher.

To investigate whether this HLA-A*0201 binding was sufficient to activate CTL lines, peripheral blood mononuclear cells from HLA-A*0201⁺ donors were restimulated weekly *in vitro* with a mixture of peripheral blood mononuclear cells separately charged with the heptamer, octamer or nonamer. Subsequently, the CTLs were stimulated with T2 cells exogenously loaded with the heptamer, the octamer or the nonamer peptide. Only the PSCA nonamer but not the heptamer or the octamer was able to stimulate the resulting CTL line to secrete interferon (IFN)- γ (Fig 2B). Taken together, cleavage of PSCA_{14–22} at positions 15 and 20 destroyed the epitope and interfered with HLA-A*0201 stabilization and recognition by CTL.

PSCA is rapidly cleaved by ER signal peptidase

The ER signal peptidase cleaves off most of the ER leader peptides as soon as the polypeptide is inserted into the lumen of the ER (Martoglio & Dobberstein, 1998). To find out how fast the signal sequence is cleaved from PSCA, we carried out short-term ³⁵S-Met/Cys metabolic labelling experiments with HEK293-PSCA-HA cells. To distinguish uncleaved and cleaved PSCA-HA, anti-HA immunoprecipitates were separated on Schaeffer gels (Schaeffer & Jagow, 1987). Short-term metabolic labelling of the full-length PSCA-HA protein and an ER-leader-deficient variant showed that both proteins could be electrophoretically separated with this gel system (Fig 3, left panel). PSCA-HA was visualized by

Fig 1 | Mapping amino-terminal signal sequence cleavage of PSCA using tandem mass spectrometry and Edman sequencing. (A) Prediction of cleavage of PSCA-HA by signal peptide peptidase according to the program Signal P-NN. The PSCA_{14–22} nonameric epitope is marked. (B,C) Analysis of PSCA by tandem mass spectrometry (LC-MS/MS). MS/MS spectra of (B) peptide LLCYSCK_{21–27} derived from the mass (M + 2H)²⁺ 472.6 (expected mass: 942.5; observed mass: 942.4) and (C) PSCA_{15–52} derived from peptide (M + 3)³⁺ 1440.1 (expected mass: 4,317.3; observed mass: 4,316.0) are shown. (D) Edman degradation of the PSCA-HA N terminus shows the sequence LLCYS... HPLC profiles of PTH standards and of the products of the first five cycles are shown. HA, haemagglutinin; HPLC, high-performance liquid chromatography; PSCA, prostate stem cell antigen; PTH, phenylthiohydantoin.



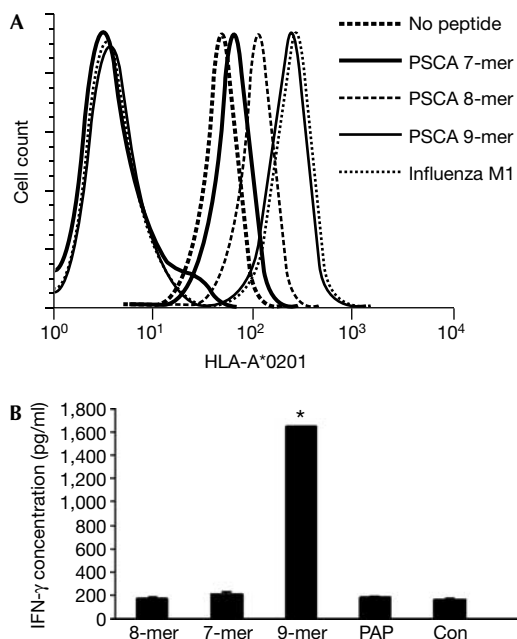


Fig 2 | Only the PSCA_{14–22} nonamer stabilizes HLA-A*0201 and achieves efficient CTL stimulation *in vitro*. (A) For an HLA-A*0201 stabilization assay, T2 cells were externally loaded with 10 μM of PSCA hepta(7-)mer (PSCA_{14–20}, thick straight line), octa(8-)mer (PSCA_{15–22}, thin dashed line) and nona(9-)mer (PSCA_{14–22}, thin straight line) peptide as well as influenza virus matrix protein epitope M1 (dotted line) as a positive control and no peptide (dashed line) as a negative control. Subsequently, HLA-A*0201 cell-surface expression was determined by flow cytometry. Corresponding stainings with an irrelevant isotype-matched FITC-conjugated control antibody are shown as an overlay on the left. (B) Only the PSCA 9-mer can be used to generate a CTL line. Human CD8⁺ T cells generated by restimulation with an equimolar mixture of PSCA 9-mer, 8-mer and 7-mer were co-incubated with T2 cells separately loaded with 9-mer, 8-mer or 7-mer. T2 cells loaded with an HLA-A*0201-restricted prostate acidic phosphatase epitope (PAP) and untreated T2 cells (Con) were used as negative controls. Activation of CD8⁺ T cells was assessed by measurement of IFN-γ release into the supernatant by ELISA. The means of triplicates and s.e.m.s are shown. The experiments were repeated twice with a similar outcome. An asterisk indicates that the data point was higher than the highest standard. CTL, cytotoxic T lymphocyte; FITC, fluorescein isothiocyanate; HLA, human leukocyte antigen; IFN-γ, interferon-γ; PSCA, prostate stem cell antigen.

autoradiography either immediately after a 5-min pulse period or after 10, 30, 60 and 120 min of chase (Fig 3, right panel). Before electrophoretic separation, the immunoprecipitates were treated with peptidyl-N-glycanase to monitor the size of PSCA-HA in the absence of glycosyl chains. Full-length PSCA-HA could be detected with a molecular mass of approximately 12 kDa immediately after synthesis, but already after 10 min of chase the protein migrated at an apparent molecular mass of approximately 10 kDa, which probably represents the mature PSCA-HA protein from which the 2.0 kDa ER leader had been removed. Therefore, the full-length pro-form of PSCA-HA did not accumulate in the ER but was rapidly cleaved by signal peptidase shortly after co-translational insertion. Consequently, uncleaved PSCA

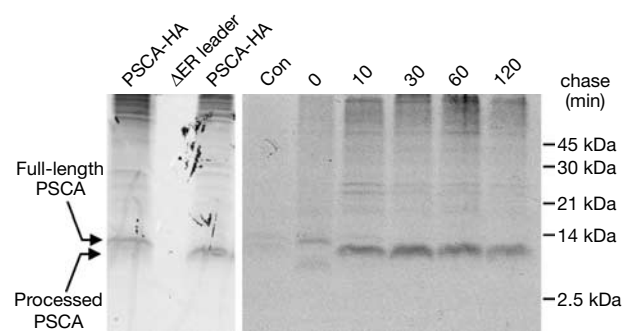


Fig 3 | The endoplasmic reticulum leader of PSCA is cleaved rapidly after synthesis. Left panel: HEK293 cells expressing full-length PSCA-HA and PSCA-HA lacking the ER leader were pulse labelled with [³⁵S]methionine/cysteine for 5 min. Right panel: HEK293-PSCA-HA cells were similarly pulse labelled for 5 min and chased for the indicated time periods. Proteins were then immunoprecipitated with anti-HA antibody, and the precipitate was deglycosylated by PNGase F before separation on a Schaeffer gel and visualization by autoradiography. The experiment was repeated twice with a similar outcome. ER, endoplasmic reticulum; HA, haemagglutinin; PSCA, prostate stem cell antigen.

containing an intact PSCA_{14–22} epitope was not available for retrotranslocation and processing in the ERAD-linked pathway.

PSCA_{14–22} relies on proteasome and TAP activity

Next, we investigated whether presentation of PSCA_{14–22} depends on proteasome and TAP activity. HEK293-PSCA-HA cells were infected with recombinant vaccinia virus (rVV) expressing the HLA-A*0201 molecule. At 1 h after infection, the proteasome was inhibited by treating the cells with 5 μM MG132 for 3 h. Subsequently, presentation of the PSCA_{14–22} epitope was assessed by determination of CTL-derived IFN-γ in the supernatant. As shown in Fig 4, proteasome inhibition reduced presentation of PSCA_{14–22} to background levels.

The cytosolic binding site of human TAP is blocked by the ICP47 protein of herpes simplex virus (HSV)-1. To examine whether the presentation of PSCA_{14–22} is TAP dependent, we infected HEK293-PSCA-HA cells simultaneously with rVV-HLA-A*0201 and rVV-ICP47 or with rVV-HLA-A*0201 and the irrelevant rVV-GP33 (expressing the lymphocytic choriomeningitis virus glycoprotein epitope GP33) as a control. Infection of HEK293-PSCA-HA cells with rVV-HLA-A*0201 was necessary, because the endogenous HLA-A*0201 expression of HEK293-PSCA-HA cells was too low. As shown in Fig 4, the presentation of PSCA_{14–22} was markedly inhibited after co-infection with rVV-ICP47, but inhibited only marginally when the cells were co-infected with the irrelevant rVV-GP33, thus indicating that TAP activity is required for presentation of PSCA_{14–22}.

The effect of blocking retrotranslocation on PSCA_{14–22}

To corroborate our conclusion that the generation of PSCA_{14–22} does not rely on the ERAD pathway, we assessed the presentation of PSCA_{14–22} in the presence and absence of the *Pseudomonas aeruginosa* exotoxin A, which has been shown to inhibit retrotranslocation in intact cells (Ackerman *et al*, 2006). To test the effect of the exotoxin A, we assessed the presentation of the

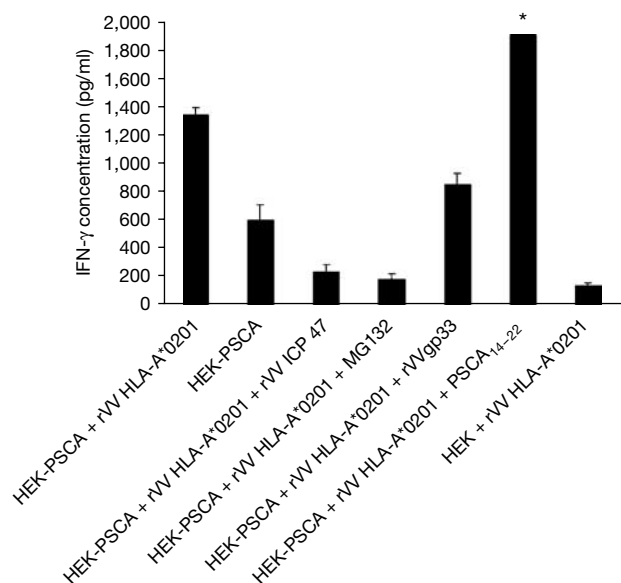


Fig 4 | Processing of the PSCA nonamer is proteasome and TAP-dependent. HEK293-PSCA-HA cells used as APCs (HEK-PSCA) were infected with rVV-HLA-A*0201 and either co-infected with rVV-ICP47 or incubated with the proteasome inhibitor MG132 (3 h, 5 μ M). PSCA₁₄₋₂₂/HLA-A*0201-specific CD8⁺ T cells were added for 18 h and the IFN- γ content in the supernatant was determined by ELISA. HEK293-PSCA-HA cells, HEK293-PSCA-HA cells infected solely with rVV-HLA-A*0201, HEK293-PSCA-HA cells co-infected with rVV-HLA-A*0201 and rVV-GP33 encoding the irrelevant lymphocytic choriomeningitis virus glycoprotein epitope GP33 acted as controls. HEK293-PSCA-HA cells infected with rVV-HLA-A*0201 and externally pulse labelled with PSCA₁₄₋₂₂ nonamer acted as a positive control; an asterisk indicates that the data point was higher than the highest standard. Untransfected HEK293 cells infected with rVV-HLA-A*0201 acted as a negative control. The means of triplicates and s.e.m.s are shown. The experiment was repeated twice with similar outcome. APC, antigen-presenting cell; HA, haemagglutinin; HLA, human leukocyte antigen; IFN- γ , interferon- γ ; PSCA, prostate stem cell antigen; rVV, recombinant vaccinia virus; TAP, transporter associated with antigen processing.

HLA-A*0201-restricted tyrosinase epitope YMNGTMSQV, the presentation of which has been shown to be dependent on ERAD (Mosse *et al*, 1998). Consistently, untreated HEK293 cells infected with a recombinant modified vaccinia virus strain Ankara, which encodes human tyrosinase (MVA-hTyr), stimulated only CTL lines specific for the peptide YMDGTMSQV with an aspartic acid at position 371 and not CTL lines specific for the peptide YMNGTMSQV with an asparagine at position 371 (Fig 5A). However, exotoxin A treatment markedly lowered stimulation of the aspartic-acid-specific CTL line and enabled prominent stimulation of the asparagine-specific CTL line, suggesting that ERAD-dependent processing was suppressed. Treatment of cells expressing PSCA-HA with exotoxin A, by contrast, had no marked effect on presentation of the PSCA₁₄₋₂₂ epitope (Fig 5B), strongly suggesting that the processing of this epitope did not involve the ERAD pathway.

To strengthen further the conclusion that processing of the PSCA-HA protein occurred outside the ER, we inserted

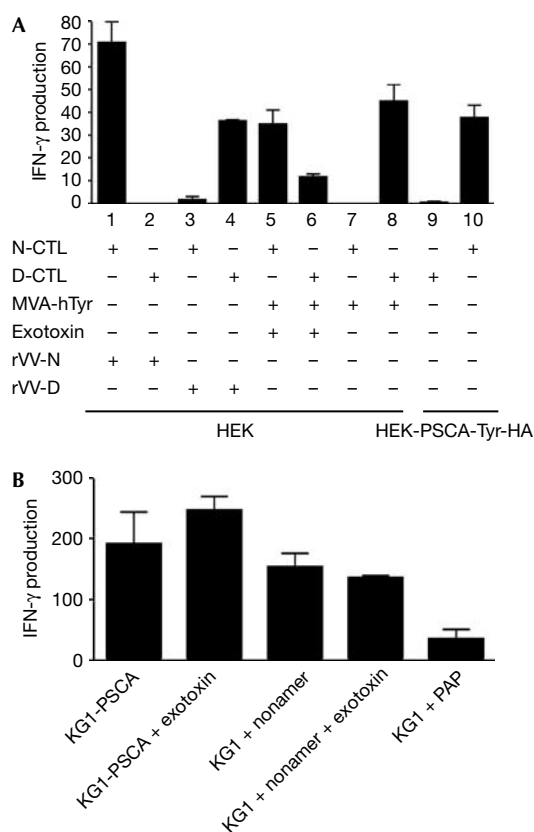


Fig 5 | Processing of PSCA occurs outside the endoplasmic reticulum and is not abrogated by the blockade of retrotranslocation. (A) HEK293 cells stably expressing the PSCA-Tyr-HA protein containing the YMNGTMSQV epitope of tyrosinase were used to stimulate a CTL line specific for the peptide YMNGTMAQV (column 10) and a CTL line specific for the peptide YMDGTMAQV (column 9). The concentration of interferon- γ (IFN- γ) in the supernatant of the CTL as determined by ELISA is shown. As a positive control for the effect of exotoxin A, HEK293 cells were infected with a recombinant modified vaccinia virus strain Ankara, which encodes human tyrosinase (MVA-hTyr), and were either untreated or treated with exotoxin A as indicated below the figure. The cells were then tested for stimulation of CTL specific for the peptide YMNGTMAQV (lanes 5,7) or the peptide YMDGTMAQV (lanes 6,8). HEK293 cells transfected with vaccinia virus encoding the YMNGTMAQV epitope (rVV-N) and the YMDGTMAQV epitope (rVV-D) acted as mutual controls for the specificity of the CTL lines (columns 1-4). (B) The dendritic cell line KG1 was transiently transfected with a PSCA-HA expression construct and the cells were either left untreated or treated with exotoxin A as indicated. External pulse labelling with the synthetic PSCA₁₄₋₂₂ nonamer was carried out to exclude a potential short-term effect of exotoxin A on MHC class I cell-surface expression. As a negative control, KG1 cells were externally pulse labelled with an irrelevant HLA-A*0201 binding peptide (PAP). The cells were incubated with a PSCA₁₄₋₂₂-specific CTL line and secretion of IFN- γ into the supernatant was assessed. The experiments were carried out twice with a similar outcome. CTL, cytotoxic T lymphocyte; ER, endoplasmic reticulum; HA, haemagglutinin; PSCA, prostate stem cell antigen.

the tyrosinase sequence ALHIYMNGTMSQVQG containing the underlined epitope in between PSCA and the HA tag. Stable transfectants of HEK293 cells expressing this PSCA-Tyr-HA protein were well recognized by YMNGTMSQV-specific CTL but not by YMDGTMSQV-specific CTL (Fig 5A). This indicates that the PSCA-Tyr-HA protein was processed outside the ER as conversion of asparagine to aspartic acid as a consequence of *N*-glycosylation in the ER.

In summary, the identification of the signal peptidase cleavage site at position 20 of the PSCA protein, which corresponds to position 7 of the nonameric epitope PSCA_{14–22}, implies that this epitope cannot be generated from PSCA polypeptides that are co-translationally inserted into the ER, because this would lead to rapid destruction of the epitope by the ER signal peptidase. Consequently, the entire processing pathway of this epitope must occur outside the ER, probably in the cytoplasm, as suggested by the requirement for proteasome and TAP activity (Fig 4). This new processing pathway differs from the current view summarized by J. Yewdell in *Immunological Reviews* in 2005: 'Indeed, the limited evidence available suggests that ER to cytosol translocation, not failure of ER import (which also generates DRiPs), is the major processing pathway for ER proteins'. Recently, it was shown that deamidation of asparagine can occur in certain cells independently of *N*-linked glycosylation by a so far poorly characterized deamidation process in the cytosol (Altrich-VanLith *et al*, 2006). This implies that the conversion from asparagine to aspartate cannot be used as sole evidence that a protein has been glycosylated in the ER, and that the extent of antigen processing through the ERAD pathway might have been overestimated.

The ERAD-independent pathway described in this work is reminiscent of the defective ribosomal products pathway along which epitopes are generated from polypeptides that are degraded shortly after their biosynthesis. It is possible that errors in the translation of ER signal peptides interfere with binding to the signal-recognition particle, which usually slows down protein synthesis until the ribosome has docked onto the translocon for co-translational insertion into the ER. In addition, the fidelity of ER targeting might be inherently low. Depending on the properties of the ER signal peptide, the efficiency of ER targeting ranges from >95% to as low as <60% of the synthesized proteins (Levine *et al*, 2005). This surprisingly low efficiency, which is close to the DRiP rate of approximately 30%, might guarantee an ample supply of protein substrates for the ERAD-independent epitope-processing pathway. In that sense, the ERAD-independent pathway described here would be a further example of how the immune system has exploited the inefficiency of cell biology for antigen processing.

METHODS

Pulse–chase analysis. HEK293-PSCA-HA cells were metabolically labelled with [³⁵S]methionine/cysteine for 5 min and chased for indicated time periods. Immunoprecipitation was carried out with an anti-HA immunoaffinity matrix (Sigma, Munich, Germany). Immunoprecipitates were deglycosylated overnight with PNGase F and separated on 16.5% Tricine–SDS–polyacrylamide gel electrophoresis (Schaeffer & Jagow, 1987) for autoradiography.

Determination of antigen presentation. Human CTL lines were generated as detailed previously (Dannull *et al*, 2000). Antigen specificity of CTL lines was determined using T2 cells as antigen-presenting cells (APCs) externally loaded with 10 µg/ml peptide for

2 h or cells transfected with rVV-N or rVV-D (Mosse *et al*, 1998) for 4 h at a multiplicity of infection (MOI) of 10. Also infection of cells with rVV-ICP47, rVV-GP33, rVV-HLA-A*0201 or MVA-hTyr was carried out for 4 h at an MOI of 10. MG132 was applied at a concentration of 5 µM for 3 h in parallel to the virus infection. Where indicated, cells were preincubated with 10 µg/ml exotoxin A (Calbiochem, La Jolla, CA, USA) for 18 h. MG132 was used at a concentration of 5 µM for 3 h in parallel to the virus infection. All antigen-presenting cells (APCs) in Fig 5 were infected with rVV-HLA-A*0201. Co-incubation of APCs and CTL lines was carried out at a ratio of 5 × 10⁴ CTL to 1 × 10⁴ APC in 96-well plates. After 18 h of co-incubation, supernatants were analysed for IFN-γ content using a human IFN-γ ELISA kit (BD OptEIA™, BD Biosciences, Basel, Switzerland).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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COMPETING INTEREST STATEMENT

The authors have no conflicting financial interests.

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